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MOLECULAR DETECTION OF METHICILLIN RESISTANCE STAPHYLOCOCCUS AUREUS FROM HORSES IN EGYPT

BY

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ABSTRACT

Staphylococcus aureus (S. aureus) is a Gram positive organism that serves as an opportunistic pathogen and frequent colonizer of the epithelium causing severe diseases in human and animals. The widespread use of antibiotics both in human and veterinary medicine resulted in the emergence of resistant strains of *S. aureus*. This resistance is determined by the *mecA* gene, which encodes the low-affinity penicillin-binding protein (PBP 2). MRSA infection was first considered hospital-associated (HA-MRSA) and community-associated MRSA (CA-MRSA) infections. However, another group emerged known as livestock-associated MRSA (LA-MRSA). The isolation of MRSA from different species, food products and the environment raised concern on the role of animals particularly livestock and wildlife in the epidemiology of MRSA.The spatial distribution of MRSA indicates interspecies transmission and colonization of different populations. The purpose of this study was to detect the prevalence of MRSA from the nostril and wound of horses. Samples were collected from nostrils and wounds of 92 horses. The isolation and identification of MRSA were carried out on selective media (Mannitol Salt agar and Blood agar) and confirmed using series of biochemical tests. The isolates were also tested for the presence of *mecA* gene. A high proportion of MRSA (61.2%) MRSA were obtained from the samples.Antibiotic sensitivity tests (AST) demonstrated the multi-resistance characteristics of the MRSA isolates.

Keywords:

MRSA, PCR, *mec-A* gene, Horse, Human, antibiotic sensitivity.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a type of bacteria that is resistant to certain antibiotics including methicillin, oxacillin, penicillin and amoxicillin **(Wichelhaus *et al.,* 1997).** *S. aureus* remains one of the most intensively investigated bacterial species of human and animal pathogens. It can cause a variety of nosocomial and community-acquired infections ranging from minor skin abscesses to serious potentially life-threatening diseases

such as bone and soft tissue intra-surgical infections, sepsis and invasive endocarditis **(Chambers, 2001; Enright *et al.,* 2002).** The epidemiology of MRSA is dynamic **(Karchmer,2008 and Blanc *et al.,* 2007).** First identified in the 1960s, MRSA was initially considered a nosocomial pathogen. Beginning in the late 20th century, a specific clone of MRSA, known as USA 300, emerged as a leading cause of community-acquired infection **(Moran *et al*., 2006; Daum, 2007)**. Recently, another strain of MRSA, Sequence Type 398 (ST-398), has been shown to be strongly associated with livestock **(Smith and Pearson, 2011)** accounting for up to 20% of all human cases of MRSA infection in the Netherlands **(van Loo *et al.,* 2007**). During this time, a growing number of reports have described probable transmission of *S. aureus* and MRSA, in particular, between humans and companion animals **(Scott *et al.,* 1988; Baptiste *et al.,* 2005)**.

Little is known, however, about the potential role of companion animals in the transmission of MRSA to humans. For example our understanding regarding direction of transmission, persistence of colonization, rate of animal-human transmission, inter-specie transmission risk factors, animal population or breeds with increased risk to be carriers of MRSA and the significance of companion animals as reservoirs for human MRSA infections are all incomplete.

MRSA in horses was first discovered in mares with metritis and stallions with dermatitis in Japan **(Anzai *et al.,* 1996)**. Later, it was reported in a Michigan veterinary hospital in 1997 with a case report of a post-operative wound infection followed by first emergence of nosocomial outbreak in 1999 **(Seguin *et al.,* 1999).** Other reports of equine infections arises but the number of cases reported are limited **(Weese *et al.,* 2005)** were the first to study community-acquired MRSA (CA-MRSA) colonization in horses following detection of a cluster of MRSA cases at a veterinary teaching hospital. Since then, MRSA has clearly emerged as an important pathogen in causing both colonization and infections in horses and personnel **(Weese *et al.,* 2005).**

Clinical MRSA infections in horses have not been as well characterized as those in human, but they can damage a broad range of tissues in this species such as soft tissue infection (STI), dermatitis, osteomyelitis,septic arthritis,omphalophlebitis,metritis, pneumonia, and septicemia and catheter site infection. The horse's nature seems to put them at risk for traumatic injuries hence wounds are common in horses. Among the major pathogens present in horses' wounds is *S. aureus*. Therefore, the presence of MRSA in such wounds may complicate and delay the

process of wound healing **(Patel, 2007).** Conventional methods (Culture, biochemical tests and antimicrobial susceptibility) are limited and time consuming. On the other hand, PCR appears to be a rapid, sensitive, and specific assay for *mec-A* gene **(Geha *et al.,* 1994; Murakami *et al.,* 1991)** determined the resistance to methicillin by the *mec-A* gene, which allows a bacterium to be resistant to antibiotics such as methicillin, and other penicillin like antibiotics, because it does not allow the ring like structure of penicillin-like antibiotics to attack the enzymes that help forming the cell wall of the bacterium, and hence the bacteria is allowed to replicate normally. Alternative medicine treatments have been practiced around the world, because the effectiveness of the currently available antibiotics has been decreased due to the increased number of resistant strains causing infections including MRSA.

The aim of the present work was the isolation and biochemical characterization of MRSA as well as detection of *mec-A* gene using PCR from companion animal especially horses. Moreover, the MRSA sensitivity patterns were checked against different antibiotics.

MATRERIAL AND METHOD

Samples:

Samples were collected from 92 horses from Animals Brooke Hospital and El Zahra station for the pure Arab horses. The samples included nostril swabs and swabs from wounds on the face, neck and limbs using sterile transport swabs containing sterile saline (Table 1)

Bacteriological studing:

Swabs were collected under aseptic conditions and cultivation of samples, isolation and purification of the isolates were carried out using different media (HIMEDIA). Swabs were inoculated into a tube containing tryptic soy broth. The broth was incubated at 37ºC for 24 hrs then streaked from the enriched broth onto mannitol salt and blood agar plates. Identification of the isolates include morphological examination by Gram's staining **(Cruickshank *et al.,* 1975).** Biochemical identification was carried out according to (**Collee *et al.,* 1996)** including catalase and coagulase tests.

Identification and characterization of *S. aureus*:

Isolates were streaked onto mannitol salt agar and incubated aerobically at 35°C for 48 hrs. Colonies identified as *S. aureus* were diagnosed according to **Bottone *et al.* (1984).**

Antibiotic Sensitivity Test:

Antibiotic resistance profile for each MRSA isolate was determined using the disc diffusion methods according to CLSI **standards (2019).**Ten antibiotics were chosen based on their common use in research, human medicine and veterinary practice. They belonged to the following groups: cephalosphorin (Cefoxitin CX), b-lactams (oxacillin OX), lincosamides **(**Clindamycin CD), macrolides **(**Erythromycin E), aminoglycoside **(**Amikacin AK), fluoroquinolones **(**Ciprofloxacin CIP), tetracycline (Tetracycline TE), sulpha/trimethoprim SXT, vancomycin VA, Polymyxi- B (PB). The percentages of sensitive, intermediate and resistant are shown in (Table 2).

PCR for identification of MRSA isolates:

Pure young cultures of the suspected MRSA isolates in addition to a standard strain of MRSA (ATCC#43300)were harvested from agar plates.Genomic DNA was extracted using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer instructions.

The *mecA* gene was amplified with the primers 5-GTT GTA GTT GTC GGG TTT GG-3 (Upstream) and 5-CTT CCA CAT ACC ATC TTC TTT AAC-3 (downstream) specific for the *mecA* gene. The PCR amplification reaction was performed according to the following thermal profile: 30 cycles of 1 min at 95^{0}C, 1 min at an annealing temperature ramped from 65 to 55^{0}C during the first 10 cycles and 1 min at 72^{0}C. The PCR product was visualized on a 1.5% agarose gel using ethidium bromide staining and a UV Tran illuminator **(Wielders *et al.,* 2002).**

RESULT AND DISCUSSION

Traditional analysis of 92 samples collected from horses including: 58 males and 34 females (20 wound and 72 nasal swabs) (Table 1) Using bacterial isolation and biochemical identification. Results revealed the presence of Gram positive, non-spore forming cocci, arranging in irregular clusters. The colonies were circular, smooth and glistening. On blood agar, they were Beta-hemolytic Fig. (1). Biochemically; the isolates positive were catalase, coagulase positive and mannitol fermenter which proved to be *S. aureus*. In vitro antibiotic sensitivity test against *S. aureus* isolates showed resistance against cefoxitin and oxacillin within 61.2% of the isolates and resistance aganist polymixin B in all isolates (Table 2). Fig. (3) Shows the electrophoretic profile of PCR for *mecA* gene confirming methicillin-resistant *S. aureus* isolates. Nosocomial infections represent a major problem in human hospitals, yet they have not been recognized as a potential problem until recently in equine veterinary hospitals. However, developments of hospitalization facilities in veterinary settings have led to opportunities for transmission of nosocomial pathogens similar to those from human hospitals.

Veterinary nosocomial outbreaks with methicillin-resistant *Staphylococcus aureus* (MRSA) were reported as early as in 1999 in the United States **(Seguin *et al.,* 1999),** followed by reports in Austria **(Cuny *et al.,* 2006 and Cuny, *et al.,* 2008),** Netherlands **(Van Duijkeren *et al*., 2010),** Sweden **(Bergström *et al.,* 2012),** Israel **(Schwaber *et al.,* 2013)** and Japan **(Kuroda *et al.,* 2016).** The present study was carried out to investigate the cause of development of multiple drug resistant strains as side infection following surgical operations in Egyptian hospital. Results showed that the outbreak was due to infection with multiple drug resistant *S. aureus* (MRSA). Results agree with **Hudson (1994) and Cookson (1998**) who proved that, the treatment of *S. aureus* infections may be complicated by multiple antibiotic resistances and specific virulence factors, causing temporary or long-lasting carriage. The nasal carriage of MRSA is a main risk factor for community-acquired infections and in hospital settings (nosocomial sepsis. Results also agree with **Gomez-Lucía *et al.,* (1989); Kloos and Bannerman (1999)** who mentioned that *S. aureus* is an opportunistic pathogen which can cause diseases ranging from superficial soft-tissue infections to life-threatening bacteremia and toxic shock syndrome. These findings agree with **Quinn *et al.,* (2002)** who accused MRSA of being a critical pathogen responsible for a great morbidity and mortality especially among immunosuppressed cases. Antibiotic sensitivity test showed resistant against cefoxitin and oxaicllin in 61.2% of *S. aureus* isolates. These results agree with **Quinn *et al.* (2002)** who proved that MRSA either produce potent toxins or resist a wide range of antibiotics.**Tiwari *et al.* (2009)** compared the performances of four phenotypic tests used to detect methicillin resistant *S. aureus* (MRSA) with the *mec-A* gene polymerase chain reaction. Recent studies reported 41.3% MRSA in equine wounds in Germany **(Vincze *et al.,* 2014) and** later 66.7% in equine wounds in Malaysia **(Nuradilah *et al.,* 2015)**. The significant high incidence of MRSA in equine wound swabs in this study clearly shows the prominent clinical importance of this pathogen in horses. Earlier studies have reported variable occurrence of MRSA.At the Ontario Veterinary College Teaching Hospital, the prevalence rate was 5.3% **(Weese *et al.,* 2006).** Later, **(Van Den Eede *et al*., 2009)** reported that MRSA was found in 10.9 % of horses presented at a Belgian equine clinic.In contrast, no colonized horses were identified during screening of healthy horses in the Netherlands **(Busscher *et al.,* 2006)** and Denmark **(Bagcigil *et al.,* 2007).** However, MRSA was present in the general horse population, based on the diversity of MRSA isolates found in hospitalized horses **(Van Den Eede *et al.,* 2009**

and Weese *et al.*, 2006).Thus, the presence of MRSA could be detected at various rates and present or colonized in horses of another country.

Table (1): number of nasal and wound horse samples against the recovered *S. aureus* and MRSA isolates.

	Nasal samples			Wound samples		
	No.	*S. aures*	MRSA	No.	*S. aures*	MRSA
Male	46	9	6	12	4	2
Female	26	4	3	8	1	-
Total	72	13	9	20	5	2

Table (2): Antibiogram results for *S. aureus* isolated from horse samples (92 samples).

Antimicrobial agent	Conc. of disk	Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
cefoxitin	30 µg/ml	7	38.8%	0	0.00	11	61.2%
oxacillin	5 µg/ml	7	38.8%	0	0.00	11	61.2%
clindamycin	2 µg/ml	18	100%	0	0.00	0	0.00
erythromycin	15 µg/ml	18	100%	0	0.00	0	0.00
Amikacin	30 µg/ml	15	83.4%	3	16.6%	0	0.00
ciprofloxacin	5 µg/ml	18	100%	0	0.00	0	0.00
Tetracycline	30 µg/ml	13	72.2%	3	16.6%	2	11.2%
Sulpha/trimethoprim	23.75+1.25 µg/ml	14	77.8%	4	22.2%	0	0.00
Vancomucin	5 µg/ml	18	100%	0	0.00	0	0.00
Polymyxin B	300units/ml	0	0.00	0	0.00	18	100%

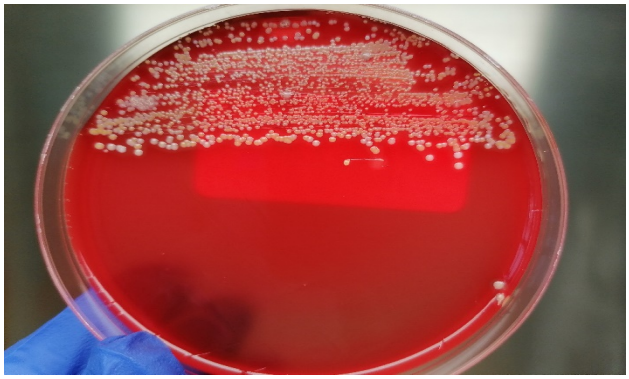

Fig. (1): MRSA strain isolated from a nasal swab showing golden yellow, Beta hemolytic colonies.

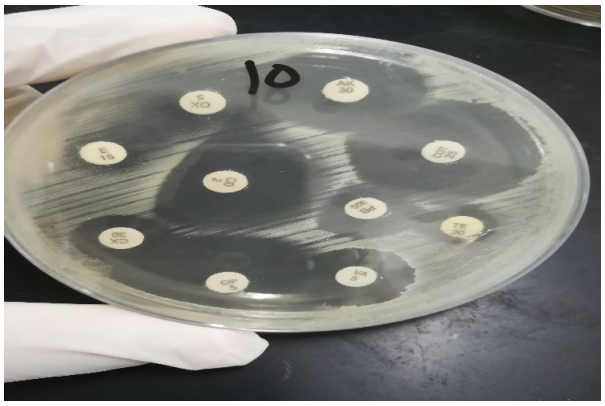

Fig. (2): MRSA strain isolated from pus (neck region) showing variable sensitivity resistance against antibiotic discs.

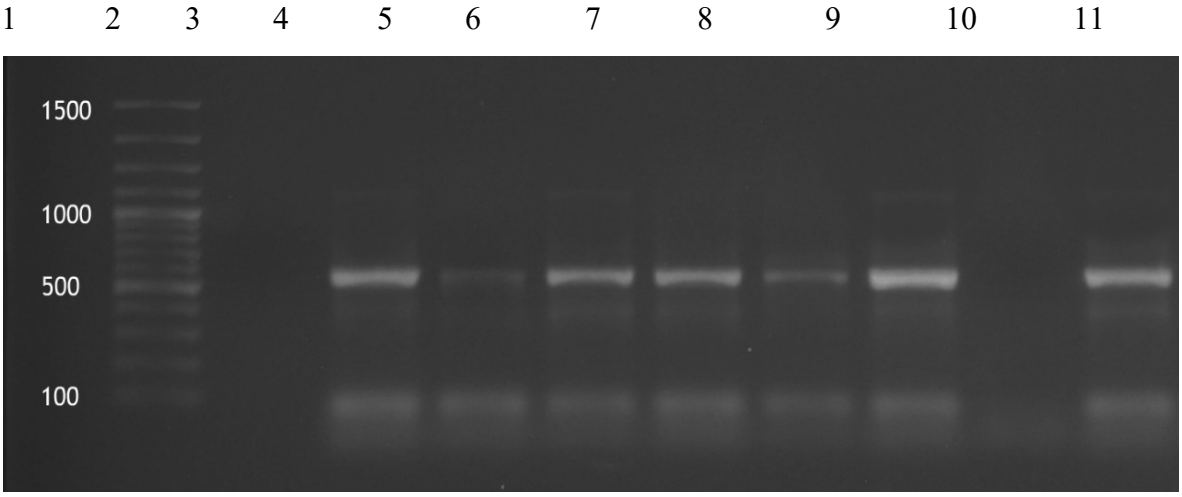

Fig. (3): Electrophoretic profile of PCR for *mecA* gene in methicillin resistant *Staphylococcus aureus*.

CONCLUSION

The prevalence of MRSA isolates in hospitals, community, animals and their products has increased in different geographical locations. The continuous vigilance of MRSA through monitoring of newer strains, their characteristic, host specificity and transmission routes in each of the settings (HA-MRSA, CA-MRSA, LA-MRSA) will help in effective control of MRSA. MRSA is no longer infection acquired in the hospital alone, but rather in communities through contact with domesticated and wild animals, as well as, food products and the environment. Therefore, there is on urgent need for effective control of MRSA in all the settings and the avoidance of indiscriminate use of antibiotics to prevent further selection of resistance by microorganisms.

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